Comparison of high temperature resistance in two buckwheat species *Fagopyrum esculentum* and *Fagopyrum tataricum*

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Summary

In the context of ongoing climate change, expected temperature rise may significantly limit plant growth and productivity of crop species. In this study, we investigated the effects of a sub-optimal temperature on buckwheat, a pseudocereal known for its nutraceutical advantages. Two buckwheat species differing by their reproduction method, namely *Fagopyrum esculentum* and *Fagopyrum tataricum* were grown at 21°C and 27°C in growth chambers. High temperature increased leaf production mainly in *F. tataricum* but decreased leaf area in both species. Water and photosynthesis-related parameters were affected by high temperature but our results suggested that although transpiration rate was increased, adaptive mechanisms were developed to limit the negative impact on photosynthesis. High temperature mainly affected the

reproductive stage. It delayed flowering time but boosted inflorescence and flower production. Nevertheless, flower and seed abortion were observed in both species at 27°C. Regarding flower fertility, heat affected more the female stage than the male stage and reduced the stigma receptivity. Pollen production increased with temperature in *F. esculentum* while it decreased in *F. tataricum*. Such discrepancy could be related to the self-incompatibility of *F. esculentum.* Both species increased their antioxidant production under high temperature to limit oxidative stress and antioxidant capacity was higher in the inflorescences than in the leaves. Total flavonoid content was particularly increased in the leaves of *F. esculentum* and in the inflorescences of *F. tataricum*. Altogether, our results showed that even if high temperature may negatively affect reproduction in buckwheat, it improves its antioxidant content.

Keywords : antioxidant, common buckwheat, heat, flower fertility, Tartary buckwheat, photosynthesis

Abbreviations:

Ai: instantaneous net photosynthesis rate

AsA: ascorbate

Ci: intercellular CO_2 concentration

CCI: chlorophyll content index

DTT: dithiothréitol

DHA: dehydroascorbate

DW: dry weight

Ei: instantaneous transpiration rate

FRAP: ferric reducing antioxidant power

FW: fresh weight

φPSII :photosystemII efficiency

GSH: Reduced glutathione

GSSG: oxidized glutathione

GSHt: Total glutathione

gs: leaf stomatal conductance

MDA: malondialdehyde

NPQ: non-photochemical-quenching

pq: photochemical quenching

ROS: reactive oxygen species

TCA: trichloroacetate

WC: water content

WUEi: instantaneous water use efficiency

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Introduction

During the last few decades, the average global temperature has increased and temperature rise is expected to continue with a rise of 2.5 to 4.5°C by the end of the 21st century (IPCC, 2014; Wang et al., 2018). Temperature increase limits plant growth and productivity in many crop species (Asseng et al., 2015; Fahad et al., 2017; Wang et al., 2018). Main crop production is expected to decrease in tropical and temperate regions due to global warming (Driedonks et al., 2016). However, in cooler regions of the world, increasing temperatures could be beneficial for crop production (Challinor et al., 2014; Driedonks et al., 2016). Indeed, in these regions, the increase in temperature might alleviate low-temperature growth inhibition at the start of the growing season, allowing earlier planting of crops and longer growing seasons (Driedonks et al., 2016). Nevertheless, these beneficial impacts do not compensate the negative ones and overall impact of temperature increase on global food security is still negative (Challinor et al., 2014).

At the plant level, high temperatures alter several physiological and metabolic processes and affect both vegetative and reproductive phases (Driedonks et al., 2016; Rezaei et al., 2015; Tayade et al., 2018). The vulnerability of plants to high temperature depends upon species and genotype (Driedonks et al., 2016; Tayade et al., 2018). Increases in temperature induce heat stress when temperatures suddenly increase above the optimal growth temperature, causing stressful conditions and having negative consequences on plant growth (Driedonks et al., 2016; Rezaei et al., 2015). Temperature plays a role in nearly all aspects of crop growth and development, such as photosynthesis, respiration, transpiration, dry matter partitioning, plant development and root growth (Ahad and Reshi, 2015; Gray and Brady, 2016; Hasanuzzaman et al., 2013; Rezaei et al., 2015). For many crops, the reproductive stage appears to be even more vulnerable to temperature increase than vegetative stage (Driedonks et al., 2016; Rezaei et al., 2015). Heat during the reproductive stage may lead to flower abortion, reduced flower fertility, fertilization failure and reduced grain filling (Driedonks et al., 2016; Rezaei et al., 2015). Moreover, oxidative damage is usually a subsequent stage of most of the abiotic stresses in plants (Fahad et al., 2017). Since they are fixed, plants cannot escape and have developed over time mechanisms to avoid or tolerate these changes (Ahad and Reshi, 2015; Hasanuzzaman et al., 2013; Rezaei et al., 2015). Plant survival under heat stress depends on the ability to perceive the heat stress stimulus, generate and transmit the signal, and initiate appropriate physiological and biochemical changes (Hasanuzzaman et al., 2013). For example, in order to cope with the oxidative stress, plants usually rely on the antioxidant defense and

produce enzymatic and non-enzymatic antioxidants (Christa and Soral-Śmietana, 2008; Fahad et al., 2017; Hasanuzzaman et al., 2013). In anticipation of the expected temperature rise, it is necessary to study the impacts of high temperatures on the development, the physiology and the reproduction of plants.

In this article, the effects of a sub-optimal temperature are investigated in two buckwheat species, common buckwheat (*Fagopyrum esculentum* Moench.) and Tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn). Buckwheat is a pseudocereal known for its nutritional advantages, mainly as they do not contain gluten, are rich in valuable proteins, and present antioxidant compounds whose importance in illness prevention or treatment is increasingly recognized (Christa and Soral-Śmietana, 2008; Fabjan et al., 2003; Jacquemart et al., 2012; Small, 2017). Its cultivation required low maintenance and is well adapted to environmentally-compatible agriculture (Christa and Soral-Śmietana, 2008; Jacquemart et al., 2012; Small, 2017; Woo et al., 2016). However, buckwheat yields remain low and variable due to various physiological and ecological characteristics (Cawoy et al., 2009; Jacquemart et al., 2012; Small, 2017). The reasons can be found in its complex reproduction or its sensitivity to abiotic factors (Cawoy et al., 2006; Jacquemart et al., 2012; Small, 2017). Buckwheat is mainly cultivated in Asia and Eastern Europe and its world production has increased during the last decade to reach 3.8 megatons in 2017 (FAOSTAT, 2019; Small, 2017). *F. esculentum* contributes to most of the buckwheat production while *F. tataricum* is less cultivated (Small, 2017).

Both buckwheat species differed by their reproduction and nutritional qualities. *F. esculentum* is self-incompatible, heterostylous and produces white to pink flowers densely clustered in racemes of cymes on short pedicels that arise from the axils of the leaves (Cawoy et al., 2009; Quinet et al., 2004; Woo et al., 2016). Pin flowers have long pistils and short stamens while thrum flowers have short pistils and long stamens and pollination between both morphs is ensured by insects (Björkman, 1995; V. Cawoy et al., 2006; Woo et al., 2016). *F. tataricum* is self-compatible and produces small homostylous greenish flowers clustered in loose racemes on short pedicels that arise from the axils of the leaves (Small, 2017). Regarding nutritional qualities, *F. tataricum* produces more antioxidants, mainly flavonoids, than *F. esculentum* which gives it a bitterer taste (Fabjan et al., 2003; Zhu, 2016). The seeds of the two species have similar contents of proteins, fats, and crude fibers while *F. tataricum* seeds have a higher content of some minerals such as Se, Zn, Fe, Co, and Ni than *F. esculentum* (Zhu, 2016). Although the nutritional and health-promoting qualities of buckwheat have been well documented and buckwheat has been identified as a crop of potential use in crop improvement

programs (Chrungoo et al., 2016; Giménez-Bastida and Zieliński, 2015; Small, 2017; Zhu, 2016), its reproduction and physiology remain largely unknown. Moreover, compared with *F. esculentum*, there is a lack of systematic information on *F. tataricum* (Small, 2017; Zhu, 2016). In this work, we compare the impact of temperature on plant development, reproduction and physiology of *F. esculentum* and *F. tataricum.* The following questions were addressed: (1) Which are the physiological strategies put in place by *F. esculentum* and *F. tataricum* in response to high temperatures? (2) How is the reproductive development of these two species affected by high temperatures? (3) Are the antioxidant capacity and the antioxidant content impacted by high temperatures in these species*?*

Material and methods

Plant material and growth conditions

Seeds of *Fagopyrum esculentum* var. Darja and *Fagopyrum tataricum* var. Zlata were obtained from Pr. Dr. Ivan Kreft (University of Ljublana, Slovenia). Seeds were germinated in peat compost (DCM, Amsterdam, Netherlands) under greenhouse conditions (temperature of 22 ± 2°C, relative humidity of 65 ± 5% and 16 h-photoperiod). Two weeks after sowing, the seedlings were transplanted into 2 L pots filled with a peat compost and transferred into growth chambers under two different conditions, (i) 21 ± 1°C (control) and (ii) 27 ± 1°C (heat) with 16 plants per species and condition. Photoperiod was 16L:8D, relative humidity was maintained at 80 ± 10%. Light was supplied by Philips HPIT 400 W lamps (Philips Lighting S.A., Brussels, Belgium) and light irradiance was at 155 ± 20 µmol m^{-2} s^{-1} at canopy level (Skye Instruments Quantum Sensor quantum meter; Hansatech Instruments, Norfolk, United Kingdom). The experiment was conducted during 71 days and during this period, plants were watered every 3 days in order to avoid water stress.

Plant growth and flowering parameters

The total number of leaves (unfolded) and inflorescences per plant were counted once a week on 10 plants per treatment and species throughout the experiment. Macroscopic appearance of the inflorescence was recorded when the tepals of the first flower buds were turning white. Flowering time was assessed by the position of the node where the first inflorescence appeared (the nodes were counted acropetally, the cotyledonary node being disregarded).

Leaf area was measured on the leaf subtending the first inflorescence on 10 plants per species and treatment after 5 weeks of treatment. Leaves were scanned and the leaf area was measured using the ImageJ software.

Ten inflorescences (between the 12^{th} and 14^{th} nodes) were collected per species and treatment after 5 and 9 weeks of treatment and conserved in FAA (formaldehyde: acetic acid: 70% alcohol, 1:1:18). The number of flowers per spikelet, of flowers per inflorescence and of spikelets per inflorescence were determined under a stereomicroscope.

Five plants per species and treatment were harvested after 9 weeks of treatment and were separated in leaves, stem, inflorescences and seeds to determine fresh weight (FW). The dry weight (DW) was determined after 48 h of incubation in an oven at 72°C and the water content (WC) was calculated as: WC = [(FW – DW)/FW]*100.

Physiological parameters

Physiological measurements were performed on the 5^{th} youngest expanded leaf of 5 plants per species and treatment, after 1, 4 and 7 weeks of heat treatment. The measured parameters were chlorophyll fluorescence, chlorophyll content, stomatal conductance (gs), and gas exchange.

Chlorophyll fluorescence was monitored using a fluorescence monitoring system (FMS II; Hansatech Instruments, Norfolk, United Kingdom) according to Mathieu et al. (2014). The quantified parameters were photosystem II efficiency (φPSII), the photochemical quenching (pq) and the non-photochemical quenching (NPQ) (Maxwell and Johnson, 2000). Chlorophyll content index (CCI) was measured using a chlorophyllometer (Opti-Sciences, CCM-200) and the measurement was taken three times on the same leaf. An automatic porometer (AP4 System, Delta-T Devices) was used to measure gs on the abaxial surface of the leaf.

Gas exchange (instantaneous net photosynthesis (Ai) and transpiration (Ei) rates, intercellular CO_2 concentration (Ci)) was measured using an infrared gas analyzer (IRGA, ADC BioScientific LCI-SD system, Hoddesdon, United Kingdom). Temperature and relative humidity in the cuvette were set at 21°C, or 27°C according to the growth chamber. The instantaneous water use efficiency (WUEi) was calculated as Ai/Ei.

Flower fertility measurement

Flower fertility (pollen viability and stigma receptivity) and pollen grains per anther were determined after 5 and 9 weeks of treatment.

Two closed anthers per flower were collected from ten random flowers per treatment (from different individuals) and stored in FAA solution. To count the number of pollen grains per anther, anthers were crushed separately on a microscope slide with 10 µl Alexander's stain (Alexander, 1969) and observed under light microscope to count all pollen grains. Pollen viability was assessed using fluorescein diacetate per Dafni et al. (2005). Pollen was collected from dozens of flowers and pollen viability was determined under microscope using a minimum of 200 pollen grains per sample and 10 samples were analysed per species and treatment.

Stigma receptivity was estimated by the esterase and peroxidase activity using a colorimetric test as described by Dafni et al. (2005). Ten stigma were analysed per species, treatment and test. Stigma were observed under stereomicroscope and classified as receptive (brown coloration), partly receptive (part of the stigma was brown) and not receptive (no brown coloration).

Antioxidant parameters

Malondialdehyde (MDA, routinely used as an indicator of membrane lipid peroxidation), total antioxidant capacity (ferric reducing antioxidant power, FRAP) and concentration of phenolic compounds were determined in leaves and inflorescences of 3 plants per species and treatment after 5 and 9 weeks of treatment. Glutathione and ascorbate concentrations were determined in leaves of 3 plants per species and treatment after 9 weeks of treatment.

Malondialdehyde concentration was determined from 250 mg of fresh frozen tissue by the thiobarbituric acid reaction as described by Heath and Packer (1968). The absorbance was measured at 532 nm using a spectrophotometer (UV1800, Shimadzu, 's-Hertogenbosh, The Netherlands). The non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Total antioxidant capacity was quantified from 1 g of fresh frozen tissue according to Thaipong et al. (2006). Hydrophilic antioxidants (AOAM fraction) were extracted by methanol and hydrophobic antioxidants (AOAD fraction) were extracted by dichloromethane. The FRAP solution (300 mM acetate buffer pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution) was warmed at 37°C before using; 150 µL of sample extracts and standards (0-800 mM Trolox) were allowed to react with 2850 µL of the FRAP solution for 30 min in dark condition. Absorbances of the colored product (ferrous

tripyridyltriazine complex) were measured at 593 nm using a spectrophotometer. Results were expressed in µM TE g^{-1} FW.

Phenolic extracts were obtained by extraction of 100 mg of fresh frozen tissue with methanol 80% according to Zhang et al. (2010). Total phenolic content was determined using the modified Folin−Ciocalteu colorimetric method and absorbances were measured at 760 nm using a spectrophotometer. The standard curve ranged of 0.0−800.0 µg of gallic acid mL^{-1}. Total flavonoid content was determined by using aluminum chloride chelation method. The standard curve ranged of 0.0−50.0 µg of quercetin mL^{-1}. The absorbance was measured at 440 nm using a spectrophotometer (UV1800, Shimadzu, 's-Hertogenbosh, The Netherlands).

For ascorbate extraction, fresh frozen tissue was homogenized in 5% metaphosphoric acid solution according to Han et al. (2013). Total ascorbate (AsA + DHA) contents were determined according to Wang et al. (1991) on the basis of Fe^{3+}– Fe^{2+} reduction by ascorbate in acid solution. The ascorbate (reduced form) and total ascorbate were determined by reading absorbance at 534 nm using a spectrophotometer and using a standard curve in the range 0–10 µmol ascorbate. Dehydroascorbate concentrations were estimated from the difference of 'total ascorbate' and ascorbate concentration.

Glutathione was quantified according to Cereser et al. (2001) and both the reduced form (GSH) and the oxidized form (GSSG) were analyzed. Total gluthatione (GSHt) was extracted from 200 mg of fresh frozen tissue with 0.1 M Tris buffer (pH 8.5) and 25 mM dithiothréitol (DTT) before addition of 6% metaphosphoric acid while GSH was extracted from 200 mg of fresh frozen tissue with 6% metaphosphoric acid. The derivatization procedure with o-phtalaldehyde was performed as described by Cereser et al. (2001). Samples were injected onto a Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter, 5 µm particle size; Macherey-Nagel) maintained at 30 °C. Samples temperature was maintained at 5°C. Analyses were performed by a Shimadzu HPLC system coupled to a RF-20A fluorescence detector (Shimadzu, 's-Hertogenbosch, The Netherlands) with an excitation wavelength of 340 nm and an emission wavelength of 420 nm. The mobile phase consisted of a 50 mM Na acetate in 8% acetonitrile (A) to 70% acetonitrile (B) gradient and the flow was 0.7 $ml \cdot min^{-1}$. The elution gradient was as follow: A for 5 min, A to B gradient for 7 min, B for 5 min, B to A gradient for 5 min and finally A for 10 min. The injection volume of GSHt and GSH was 5 µL and the standard curve was 0-50µM GSH. Then, GSSG concentration was obtained from subtraction of the GSH from the GSHt values.

Statistical analyses

All of the analyses were conducted in R studio. The normality of the data was estimated using Shapiro–Wilk tests, and homoscedasticity was verified using Levene's tests. The data were transformed (logarithm or square root) when required, to ensure normal distributions. The ANOVA models were defined to evaluate the effects of the temperature, the species and their interaction on the measured parameters. When the homoscedasticity was not met, Kruskall-Wallis tests were performed. If not indicated otherwise data are presented as means ± SD.

Results

Temperature increased leaf and inflorescence production

The plastochron differed between the two species with *F. tataricum* producing more leaves than *F. esculentum* ($F_{1,36}$ = 16.71, P < 0.001, Fig. 1A). Temperature increased leaf initiation rate from the third week of treatment ($F_{1,36}$ = 7.45, P = 0.0097) and the effect of temperature was stronger in *F. tataricum* than in *F. esculentum* during the last weeks of stress ($F_{1,36}$ = 5.99, P = 0.0196). At the end of the experiment, *F. esculentum* had 41 leaves at 21°C and 48 leaves at 27°C while *F. tataricum* had 56 leaves at 21°C and 127 leaves at 27°C. The leaf area was similar in both species and decreased by 50% with the temperature (Table 1). As a result, the total leaf DW was higher at 21°C than at 27°C for *F. esculentum* ($F_{1,8}$ = 16.12, P = 0.0039) while there was no difference of total leaf DW for *F. tataricum* ($F_{1,8}$ = 4.5, P = 0.067, Table 1). The DW of the stem was higher for *F. esculentum* than for *F. tataricum* but was not affected by the temperature (Table 1).

F. esculentum flowered before *F. tataricum* as the first inflorescence appeared after production of 6.6 ± 1.0 and 9.0 ± 1.1 leaves, respectively ($F_{1,36}$ = 55.55, P < 0.001). The temperature delayed flowering time by 6% in *F. esculentum* and by 13% in *F. tataricum* ($F_{1,36}$ = 5.21, P = 0.029). Regarding inflorescence production, *F. esculentum* produced more inflorescences than *F. tataricum* at the start of the treatment but the trend was reversed from the sixth week of treatment (Fig 1B). Indeed, at the end of the experiment, *F. esculentum* had less inflorescences than *F. tataricum* (49.2 ± 20.6 vs. 96.7 ± 42.6, χ^2_1 = 15.23, P < 0.001, Fig 1B). However, the number of flowers per spikelet (χ^2_1 = 21.36, P < 0.001, Fig. 2A), of spikelets per inflorescence (χ^2_1 = 21.65, P = 0.0033) and of flowers per inflorescence (χ^2_1 = 37.23, P < 0.001, Fig. 2B) were higher in *F. esculentum* than in *F. tataricum*. High temperature increased the number of

inflorescences in *F. tataricum* but not in *F. esculentum* during the 2 last weeks of treatment (χ^2_1 = 8.7, P = 0.0032). Regarding the inflorescences, the temperature increased the number of flowers per spikelet (χ^2_1 = 11.89, P < 0.001, Fig. 2A) but did not affect the number of spikelets per inflorescence (χ^2_1 = 0.35, P = 0.853, Fig.) neither the number of flowers per inflorescence (χ^2_1 = 0.008, P = 0.926, Fig. 2B). At the end of the experiment, the total inflorescence DW (without seeds) was higher for *F. esculentum* than for *F. tataricum* ($F_{1,16}$ = 20.27, P < 0.001, Fig. 2C) and higher at 27°C than at 21°C ($F_{1,16}$ = 12.63, P = 0.0026, Fig. 2C).

Temperature decreased flower fertility

The stigma receptivity was similar in both species (71. 4% for *F. esculentum* vs. 75% for *F. tataricum,* χ^2_1 = 0.066, P = 0.8) but decreased with the temperature increase (91% at 21°C vs. 50% at 27°C, χ^2_1 = 8.77, P = 0.003). The stigma receptivity was the same whatever the floral morph in *F. esculentum* (χ^2_1 = 0.69, P = 0.41).

The number of pollen grains per anther varied according to the species with the anthers of *F. esculentum* producing twice more pollen grains than the ones of *F. tataricum* (χ^2_1 = 60.58, P < 0.001, Fig. 2D). For *F. esculentum,* the thrum flowers produced less pollen grains than the pin flowers (F = 26.99, P < 0.001). The temperature impact differed between the two species: *F. tataricum* produced more pollen grains at 21°C than at 27°C ($F_{1,38}$ = 6.18, P = 0.017, Fig. 2D) while it was the opposite for *F. esculentum* ($F_{1,76}$ = 26.74, P < 0.001, Fig. 2D). This difference was more visible in the thrum flowers ($F_{1, 38}$ = 37.73, P < 0.001). Indeed the number of pollen grains increased by 75% and 15% with the temperature in thrum and pin flowers respectively. However, the pollen viability was above 90% whatever the variety, the morph or the temperature.

Temperature affected the water status and the photosynthesis

The temperature affected the water content in a species-dependent manner (Table 1). There was no effect of temperature on the leaf and stem WC of *F. esculentum* ($F_{1,8}$ = 0.47, P = 0.51 and $F_{1,8}$ = 0.31, P = 0.59 for leaves and stems respectively), while the leaf and stem WC decreased with the temperature in *F. tataricum* ($F_{1,8}$ = 10.55, P = 0.012 and $F_{1,8}$ = 10.91, P = 0.011 for leaves and stems respectively, Table 1). Leaf stomatal conductance varied also with the temperature in a species-dependent manner (Table 2). In *F. esculentum,* gs was higher at 21°C than at 27°C the first week of stress and three weeks later, gs was higher at 27°C than at 21°C. In *F. tataricum*, gs was higher at 27°C than at 21°C whatever the date. However, the effect of temperature on gs was no more significant at the end of the experiment. Regarding the

transpiration rate, Ei was higher at 27°C than at 21°C along the experiment for both species (Table 3).

As observed for gs, the effect of temperature on the net photosynthesis and the intercellular CO_2 concentration varied along the experiment. One week after treatment, the Ai was higher at 27°C than at 21°C for the 2 species (Table 3). Three weeks later, the situation was reversed and the difference disappeared at the end of the experiment. Regarding Ci, it was higher at 21°C than at 27°C at the begin of the experiment (412.4 ± 3.9 vs. 361.7 ± 11.3 µmol mol, $\chi^2_1 = 14.3$, $P < 0.001$). Then, the difference disappeared also and Ci stayed at 388.3 ± 12.9 µmol mol^{-1}. Regarding the WUEi, the results differed among weeks (Table 4). At the beginning of the experiment, WUEi was higher at 27°C than at 21°C. Three weeks later, it was higher at 21°C than at 27°C. At the end of the experiment, WUEi was still higher at 21°C than at 27°C for *F. tataricum* but there was no more differences between treatments for F. *esculentum*.

The chlorophyll content index differed between species ($F_{1,44} = 31.2$, $P < 0.001$) and was 44.5 ± 7.2 in *F. esculentum* and 33.4 ± 4.1 in *F. tataricum.* However, it did not vary with the temperature ($F_{1,44} = 0.26$, $P = 0.61$). About the chlorophyll fluorescence, most of the effects were visible at the end of the heat treatment where φPSII (0.76 ± 0.03 vs. 0.66 ± 0.09, $F_{1,7} = 7.3$, $P = 0.03$) and pq (0.94 ± 0.02 vs. 0.8 ± 0.07, $F_{1,7} = 15$, $P = 0.006$) were higher at 21°C than at 27°C. The only impact of temperature on NPQ was observed 4 weeks after the beginning of the treatment and NPQ was higher at 21°C than at 27°C (0.35 ± 0.12 vs. 0.2 ±0.03, $F_{1,14} = 19.2$, $P < 0.001$).

Temperature increased the antioxidant content

The effect of temperature on MDA concentration varied according to the species and the date (Fig. 3 A, B). In the leaves, after 4 weeks of treatment, the concentration of MDA was higher at 27°C than at 21°C for the 2 species ($F_{1,20} = 19.55$, $P < 0.001$, Fig. 3A). It was also higher in *F. tataricum* than in *F. esculentum* ($F_{1,20} = 13.87$, $P = 0.0013$, Fig. 3A). Four weeks later, the difference between species disappeared and the temperature effect was different between the 2 species ($F_{1,22} = 20.79$, $P < 0.001$). The MDA concentration remained higher at 27°C than at 21°C in the leaves of *F. tataricum* ($F_{1,10}$ 23.31, $P < 0.001$) while it was lower at 27°C than at 21°C in the leaves of *F. esculentum* ($F_{1,12} = 7.07$, $P = 0.021$). In the inflorescences, the concentration of MDA was higher at 27°C than at 21°C ($F_{1,22} = 4.93$, $P = 0.037$, Fig. 3B) and higher in *F. esculentum* than in *F. tataricum* ($F_{1,22} = 5.87$, $P = 0.024$) after 4 week of treatment and no significant differences were observed at the end of the experiment.

The effect of temperature on the total antioxidant activity was mainly visible at the end of the experiment (Fig. 3 C-F). The total content of hydrophobic antioxidants in the leaves increased with the temperature for both species 8 weeks after treatment ($F_{1,8}$ = 6.93, P = 0.03, Fig. 3C) but no differences were observed in the inflorescences at any time for the hydrophobic antioxidant content (Fig. 3D). The hydrophilic antioxidant content in the leaves was also higher at 27°C than at 21°C at the end of the experiment but only for *F. tataricum* ($F_{1,4}$ = 28.87, P = 0.0058, Fig. 3E). Regarding the inflorescences, the concentration of hydrophilic antioxidants was higher at 27°C than at 21°C for *F. tataricum* 4 weeks after treatment ($F_{1,4}$ 12.52, P = 0.024, Fig. 3F) and for both species 8 weeks after treatment ($F_{1,5}$ = 6.19, P = 0.055).

The concentrations of phenolic compounds, flavonoids, ascorbate and glutathione were also determined 8 weeks after treatment (Fig. 4). The flavonoid concentration increased with the temperature in the leaves of the two species ($F_{1,8}$ = 105.98, P < 0.001, Fig. 4A) and in the inflorescences of *F. tataricum* (χ^2_1 = 6, P = 0.014, Fig. 4B). The polyphenol concentration in the leaves was also higher at 27°C than at 21°C for both species ($F_{1,8}$ = 70.3, P < 0.001, Fig. 4C) but it did not change with the temperature in the inflorescences ($F_{1,5}$ = 0.8477, P =0.3994, Fig. 4D). Regarding the ascorbate concentration in the leaves, the total ascorbate concentration increased with the temperature in *F. tataricum* only ($F_{1,4}$ = 35.6, P = 0.0039, Fig. 4E) while the AsA concentration increased with the temperature in both species (χ^2_1 = 5.77, P = 0.016). The AsA to DHA ratio was 2.1 ± 1.7 whatever the species and temperature. Regarding the glutathion concentration in the leaves, the GSH concentration was also higher at 27°C than at 21°C for both species ($F_{1,8}$ = 7.82, P = 0.023, Fig. 4F) while the GSSG concentration did not change with the temperature. The ratio GSSG/GSH increased with the temperature in *F. esculentum* only (χ^2_1 = 3.86, P = 0.049). However, both the GSSG concentration ($F_{1,8}$ = 8.88, P = 0.017) and the GSSG to GSH ratio ($F_{1,8}$ = 29.89, P < 0.001) were higher in *F. esculentum* than in *F. tataricum* leaves.

Discussion

Impact of temperature increase on vegetative growth

We observed that *F. esculentum* and *F. tataricum* differed by their leaf production but high temperature increased leaf initiation rate and decreased leaf area in both species. A rise in the number of leaves with temperature is a common trait in many plant species and was among others observed in *Arabidopsis thaliana*, *Solanum lycopersicum* and *Borago officinalis* (Descamps et al., 2018; Farooq et al., 2011; Giorno et al., 2013; Gray and Brady, 2016). This

strategy enables the plant to increase its transpiration surface in order to decrease leaf temperature (Prasad et al., 2008). However, in our experiment, the increase of the number of leaves at high temperature was mitigated by the decrease of the leaf area. A smaller leaf area in response to high temperature had also been observed in other studies like on *Cichorium intybus* (Mathieu et al., 2014). The small leaf area at 27°C could be explained by a reduced cellular elongation or a decrease in the number of cells (Ahad and Reshi, 2015; Machado and Paulsen, 2001). In *Cichorium intybus,* the decrease of leaf area was associated with a reduction in leaf water content due to high temperature (Mathieu et al., 2014). Our study highlighted weaker water content in stems and leaves at 27°C for *F. tataricum* but not for *F. esculentum*. However, high temperature is not always associated with a decrease of leaf area and no change or even increase in leaf area could be observed in response to high temperature (Hatfield and Prueger, 2015; Prasad et al., 2008). Finally, we observed for the two species a higher transpiration rate at 27°C during all the experiment. This strategy enables also the leaves to cool down (Rezaei et al., 2015; Shah and Paulsen, 2003). Indeed, when the stomata are open, water steam can be released which reduces the leaf temperature. It is often observed in response to heat like it was in *Solanum tuberosum* (Lizana et al., 2017).

A higher opening of the stomata can also have an impact on the gas exchanges and on the net photosynthesis rate (Lizana et al., 2017). Here, we observed at high temperature an increase in net photosynthesis rate and in intercellular CO_2 concentration at the beginning of the experiment before reaching a balance. In addition, for the water use efficiency, it was higher at 27°C the first week of the experiment and then higher at 21°C. At the end of the experiment, water use efficiency was higher at 21°C only for *F. tataricum*. Stomatal conductance was higher at 27°C mainly 4 weeks after treatment. These results suggest that buckwheat develops adaptive mechanism regarding photosynthesis with the time in response to high temperature. Increase in net photosynthesis, stomatal conductance and water use efficiency was also observed in response to high temperature in *Borago officinalis* (Descamps et al., 2018). The ability to sustain CO_2 assimilation rates and high water use efficiency under high temperature has been shown to be directly correlated with heat tolerance (Hasanuzzaman et al., 2013; Tambussi et al., 2007). Photosynthesis is one of the most heat sensitive physiological processes in plants and light-dependent photosynthetic reactions are usually more affected by high temperature than light-independent reactions (Descamps et al., 2018; Hasanuzzaman et al., 2013; Prasad et al., 2008). Regarding the light phase, the chlorophyll content was different between the two

Fagopyrum species but not between the two temperatures. An increase in temperature had well an effect on the photosystem II at the end of the experiment with a decrease of φPSII, qP and NPQ. The photosystem II is quite sensitive to heat stress as heat increases the fluidity of thylakoid membranes and disrupts metabolic processes related to electron transport (Hasanuzzaman et al., 2013; Prasad et al., 2008; Rezaei et al., 2015).

Impact of temperature increase on reproductive growth

Flower production differed between buckwheat species. In our experiment, *F esculentum* flowered earlier but produced less inflorescences than *F. tataricum*. However, *F. esculentum* had more spikelets and flowers per inflorescence than *F. tataricum* so that the total number of flowers was higher in *F. esculentum* than in *F. tataricum*. Our results confirm previous results regarding the flowering time of both species (Cepková et al., 2009). Nevertheless, Cepková et al. (2009) reported that *F. esculentum* produced generally more inflorescences per plant than *F. tataricum.* Such discrepancy could be explained by inter variety differences within a same species. Indeed, in their cultivar screening, Cepková et al. (2009) showed that the number of inflorescences per plant was highly variable in buckwheat, ranging from 5 to 81 in *F. esculentum* and from 6 to 33 in *F. tataricum* depending on the variety. We may thus not exclude that Darja produced less inflorescences compared to other *F. esculentum* varieties while Zlata produced more inflorescences compared to other *F. tataricum* varieties.

Our results showed that high temperature delayed flowering time in both species. Michiyama et al. (2001) previously reported that the first flowering node was also higher under high temperature in *F. esculentum*. High temperature is predicted to hasten flowering in several crop species (Dudley et al., 2018; Gray and Brady, 2016; Kazan and Lyons, 2016; Susila et al., 2018) but delayed flowering time under high temperature was observed in other short-day plants such as chrysanthemum (Nakano et al., 2013). Although flowering time was delayed, we observed that high temperature boosted the inflorescence and flower production in buckwheat. Indeed the number of inflorescences of *F. tataricum* has doubled with the temperature increase and the inflorescence DW was higher at 27°C than at 21°C for both species. Michiyama et al. (2001) reported a prolonged flowering period and an increased number of inflorescences under high temperature for *F. esculentum*. However, in our experiment, heat did not significantly increase the total number of inflorescences in *F. esculentum* although it increased the number of flowers

per spikelet and the inflorescence DW. It has been proposed that the strategy of *F. esculentum* under stressful conditions consists in the production of more flowers to offset the lower number of flowers at anthesis (Cawoy et al., 2009; Cepková et al., 2009; Jacquemart et al., 2012; Lachmann and Adachi, 1990). The number of flowers of *F. esculentum* at anthesis was indeed significantly lower at 27°C than at 21°C (data not shown). A similar hypothesis could be proposed for *F. tataricum* although we did not quantify the number of flowers at anthesis in this latter as most flowers are cleistogamous and did not open (Zhou et al., 2018). However, we observed flower abortion in both species in response to temperature increase. According to the literature, heat affects the inflorescence and flower production in many species (Driedonks et al., 2016; Gray and Brady, 2016; Hasanuzzaman et al., 2013) but it most often decreases the number of inflorescences and flowers produced as observed in *Borago officinalis* (Descamps et al., 2018). Flower abortion is also commonly reported in response to high temperature (Borghi et al., 2019; Descamps et al., 2018; Erickson and Markhart, 2002; Prasad et al., 2017).

Besides the impacts on flower production, high temperature can reduce the flower fertility and so the number of seeds and fruits produced (Ahad and Reshi, 2015; Descamps et al., 2018; Gray and Brady, 2016; Hatfield and Prueger, 2015; Prasad et al., 2017; Rezaei et al., 2015). The pollen viability and stigma receptivity are good indicators of the impacts of stresses on the reproduction. We observed that high temperature had no impact on pollen viability but decreased stigma receptivity in both *Fagopyrum* species, suggesting that female organs were more affected than male organs. The genus *Fagopyrum* is known for its high pollen viability and female gametophytes were shown to be much more sensitive to high temperature than male gametophytes in *F. esculentum* (Płażek et al., 2019; Słomka et al., 2017). In that respect, buckwheat differs from most plant species since the male organs are usually more sensitive to abiotic stresses than the female organs (Borghi et al., 2019; Gray and Brady, 2016; Hasanuzzaman et al., 2013; Hatfield and Prueger, 2015; Prasad et al., 2017). We also observed differences regarding the pollen production. *F. esculentum* produced more pollen grains per anther than *F. tataricum,* which is relevant with the requirement of cross-pollination of *F. esculentum* and the cleistogamy of *F. tataricum* (Wu et al., 2017). Indeed, *Fagopyrum* species differed by their reproduction strategies as *F. esculentum* is heterostylous and self-incompatible while *F. tataricum* is homostylous and self-compatible (Cawoy et al., 2009; Quinet et al., 2004; Small, 2017; Woo et al., 2016). In *F. esculentum*, pin flowers produced more pollen grains than thrum flowers which confirms the previous observations (Cawoy et al., 2009; Słomka et al.,

2017; Wu et al., 2017). The effect of high temperature on pollen production differed between species: heat increased the number of pollen grains in *F. esculentum* but decreased it in *F. tataricum.* Pollen production and development is energetically very costly (Borghi et al., 2019) and in several species pollen production decrease with high temperature due among other to a shortage in assimilate supply (Borghi et al., 2019; Descamps et al., 2018; Prasad et al., 2017; Pressman et al., 2002; Snider and Oosterhuis, 2011). We may hypothesize that, in *F. esculentum,* more resources were allocated to pollen production under high temperature to maintain successful pollination while such mechanism was not favored in *F. tataricum*. Low flower fertility will result in low seed set (Prasad et al., 2017; Rezaei et al., 2015; Soltani et al., 2019; Tayade et al., 2018). Impact of high temperature on seed set was not formally investigated in our study although we observed than the number of aborted seeds increased by more than 50% under high temperature in *F. tataricum* and heat was reported to decrease seed set in *F. esculentum* (Farooq et al., 2016; Jacquemart et al., 2012).

Impact of temperature increase on oxidative stress and antioxidant production

The production of reactive oxygen species (ROS) under high temperatures is frequently observed which leads to an oxidative stress (Hasanuzzaman et al., 2013; Liu and Huang, 2000). In our study, an increase in temperature has caused an oxidative stress but not during the whole experiment. Malondialdehyde is an indicator of membrane lipid peroxidation caused by ROS and thus an indicator of oxidative stress (Bita and Gerats, 2013; Hasanuzzaman *et al*. 2013; Liu and Huang, 2000). There was an increase of MDA with temperature 4 weeks after the start of the treatment in the leaves and the inflorescences of both species but at the end of the heat treatment, oxidative stress remained only visible in the leaves of *F. tataricum*. It suggests that antioxidant metabolism was boosted along the treatment to limit oxidative stress mainly in *F. esculentum*. To protect themselves against the production of ROS, plants can synthetize protectives compounds like antioxidants (Ramakrishna and Ravishankar, 2011). The species tolerant to high temperatures will be the ones that produce the more antioxidants (Bita and Gerats, 2013; Chaitanya et al., 2002; Hasanuzzaman et al., 2013). Under control conditions, *Fagopyrum* sp. is already known to contain a lot of antioxidants (Cepková et al., 2009; Fabjan et al., 2003; Jacquemart et al., 2012; Lee et al., 2016; Lim, 2013). Our results showed that the antioxidant capacity increased in both species in response to temperature rise. In *F. esculentum*, the increase in antioxidant capacity was mainly observed at the end of the experiment, explaining why oxidative stress was no more visible at that time. In *F. tataricum*, high

antioxidant capacity was already observed at 27°C 4 weeks after the start of the treatment. The antioxidant capacity was higher in the inflorescences that in the leaves for both species and there were more hydrophilic than hydrophobic antioxidants.

Different types of antioxidants can be produced. Here, one big antioxidant family was highlighted, the phenolic compounds which are hydrophilic antioxidants. The concentrations of polyphenols were increased by high temperature in the leaves of both species. A rise in polyphenols in response to a heat stress was also observed in other species like the tomato or the vine (*Vitis vinifera* L.) (Rivero et al., 2001; Wen et al., 2008). Even deeper in this family, there is one sub-group, the flavonoids which were also in higher concentration at higher temperature in our experiment. In the literature, flavonoids are reported as very present in *Fagopyrum* sp., mainly rutin and quercetin, and even more in *F. tataricum* than in *F. esculentum* (Cepková et al., 2009; Fabjan et al., 2003; Jacquemart et al., 2012; Lee et al., 2016; Lim, 2013). We confirmed the higher concentrations of flavonoids in *F. tataricum* compared to *F. esculentum* and the concentration of flavonoids was particularly increased in the inflorescences of *F. tataricum* in response to heat. We also quantified the concentrations of other antioxidants. Ascorbate and glutathione are antioxidants often involved in the protection against oxidative stress (Latowski et al., 2010; Noctor and Foyer, 1998; Veljović-Jovanović et al., 2017). They occur in a reaction cycle in order to reduce the hydrogen peroxide, one of the ROS that can be produced in case of oxidative stress (Latowski et al., 2010; Noctor and Foyer, 1998; Veljović-Jovanović et al., 2017). Ascorbate and/or glutathione concentrations increased with the temperature in our experiment, showing a good protection of the plants. However, our results suggested that ascorbate was mainly involved in H_2O_2 detoxification in *F. tataricum* while H_2O_2 detoxification mainly depends on glutathione in *F. esculentum.* Other species like maize, Arabidopsis and wheat have shown similar increase in ascorbate and glutathione at high temperatures (Latowski et al., 2010; Tiwari and Yadav, 2019; Veljović-Jovanović et al., 2017).

Conclusion

Altogether, our results suggest that 27°C is not a detrimental temperature for vegetative growth of *F. esculentum* and *F. tataricum* as plant growth was maintained and even boosted in *F. tataricum* and adaptive mechanisms were developed to limit the negative impact on photosynthesis. However, a temperature of 27°C affected the reproductive stage. Although,

temperature increase boosted the inflorescence and flower production, flower and seed abortion were increased, suggesting that buckwheat compensate the low flower fertility by increasing the flower production. Regarding flower fertility, heat affected more the female stage than the male stage which is surprising compared to other plant species. The impact of heat on pollen production also differed between species since *F. esculentum* preserved pollen production under high temperature which was not observed for *F. tataricum*. This difference could be explained by the difference of reproduction strategy between both species. Moreover, both species increased their antioxidant production under high temperature to limit oxidative stress and antioxidant capacity was higher in the inflorescences than in the leaves. So, even if temperature increase may negatively affect reproduction in buckwheat, it improves its nutraceutical properties.

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Figure captions

Figure 1: Temperature effect on the production of leaves and inflorescences of two buckwheat species, *F. esculentum* and *F. tataricum*. (A) Total number of leaves, (B) total number of inflorescences. The plants were cultivated at either 21°C or 27°C for 8 weeks.

Figure 2: Temperature effect on reproductive parameters of two buckwheat species, *F. esculentum* and *F. tataricum*. (A) Number of flowers per spikelet, (B) number of flowers per inflorescence, (C), inflorescence dry weight and (D) number of pollen grains per anther. The plants were cultivated at either 21°C or 27°C for 8 weeks.

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Table 1: Temperature effect on the dry weight and the water content of leaves and stems and on the leaf area of two buckwheat species. The plants were cultivated at either 21°C or 27°C for 8 weeks.

Species	Temperature	Dry weight (g)		Water content		Leaf area (cm^2)
		Leaves	Stems	Leaves	Stems	
F. esculentum	21°C	2.3 ± 0.67	8.76 ± 2,54	0.775 ± 0.035	0.818 ± 0.028	26.69 ± 10.27
	27°C	0.86 ± 0.44	6.94 ± 1,23	0.795 ± 0.054	0.808 ± 0.03	12.86 ± 7.33
F. tataricum	21°C	0.84 ± 0.46	4.54 ± 1,02	0.844 ± 0.034	0.841 ± 0.022	23.3 ± 9.13
	27°C	1.84 ± 0.95	5.78 ± 3,19	0.754 ± 0.052	0.769 ± 0.043	11.56 ± 5.62
ANOVA 2	Species	$F_{1,16}$ = 0.66 P = 0.43	$F_{1,16}$ = 7.54 P = 0.014	$F_{1,16}$ = 0.52 P = 0.48	$F_{1,16}$ = 0.35 P = 0.56	$F_{1,21}$ = 1.01 P = 0.32
	Temperature	$F_{1,16}$ = 0.55 P = 0.47	$F_{1,16}$ = 0.088 P = 0.77	$F_{1,16}$ = 3.07 P = 0.099	$F_{1,16}$ = 8.25 P = 0.011	$F_{1,21}$ = 19.45 P < 0.001
	Interaction	$F_{1,16}$ = 16.97 P < 0.001	$F_{1,16}$ = 2.44 P = 0.14	$F_{1,16}$ = 7.55 P = 0.014	$F_{1,16}$ = 4.63 P = 0.047	$F_{1,21}$ = 0.0086 P = 0.93

Table 2: Temperature effect on the stomatal conductance ($mmol.m^{-2}.s^{-1}$) of two buckwheat species. The plants were cultivated at either 21°C or 27°C for 8 weeks.

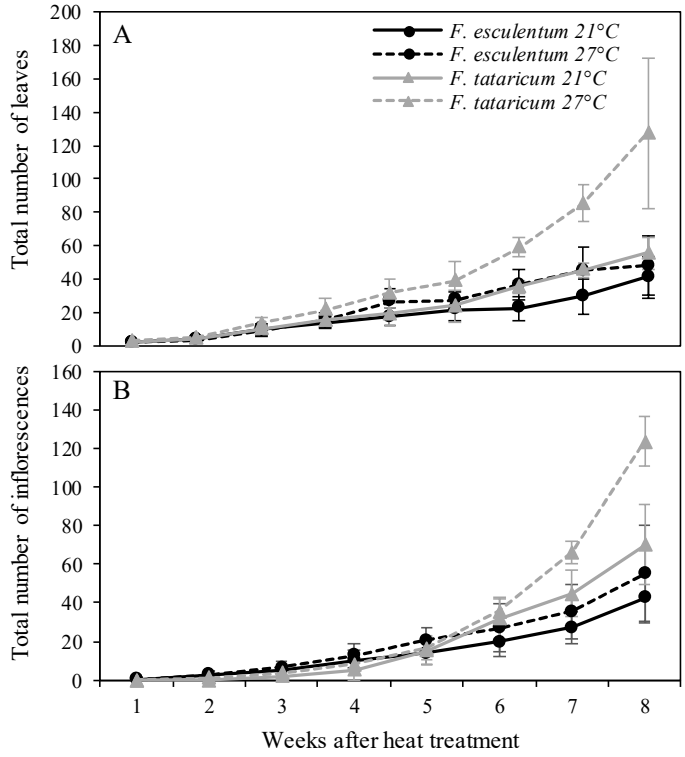
Species	Temperature	Weeks after treatment		
		Week 1	Week 4	Week 7
F. esculentum	21°C	443.75 ± 52.97	350.8 ± 94.92	139 ± 44.64
	27°C	338.75 ± 106.09	966 ± 583.03	154.33 ± 77.83
F. tataricum	21°C	341 ± 67.49	290.4 ± 97.01	87.67 ± 38.59
	27°C	536 ± 49.29	576 ± 143.8	193 ± 137.5
ANOVA 2	Species	$F_{1,14} = 1.98$ $P = 0.181$	$F_{1,16} = 2.61$ $P = 0.125$	$F_{1,8} = 0.017$ $P = 0.9$
	Temperature	$F_{1, 14} = 3.41$ $P = 0.0857$	$F_{1, 16} = 19.07$ $P < 0.001$	$F_{1, 8} = 1.53$ $P = 0.25$
	Interaction	$F_{1,14} = 19.96$ $P < 0.001$	$F_{1,16} = 0.27$ $P = 0.61$	$F_{1,8} = 0.85$ $P = 0.38$

Table 3: Temperature effect on the transpiration rate (Ei, mmole H_2O m^2/sec) and the net photosynthesis rate (Ai, µmole CO_2 m^2/sec.) of 2 buckwheat species. The plants were cultivated at either 21°C or 27°C for 8 weeks.

Parameter	Species	Temperature	Weeks after treatment		
			Week 1	Week 4	Week 7
Ei	*F. esculentum*	21°C	2.27 ± 0.77	3.15 ± 0.38	1.44 ± 0.3
		27°C	3.29 ± 1.38	5.51 ± 0.11	1.98 ± 0.24
	F. tataricum	21°C	2.36 ± 0.19	2.61 ± 0.29	1.35 ± 0.26
		27°C	3.9 ± 0.92	4.54 ± 0.36	2.2 ± 0.36
Ai	*F. esculentum*	21°C	0.84 ± 0.45	3.81 ± 0.3	1.93 ± 0.27
		27°C	3.95 ± 1.84	2.35 ± 0.64	2.39 ± 0.55
	F. tataricum	21°C	1.28 ± 0.13	3.64 ± 0.7	1.97 ± 0.43
		27°C	5.65 ± 1.53	2.21 ± 1.07	1.22 ± 0.48
Ei	ANOVA 2	Species	$F_{1.16} = 0.85$	$F_{1,16} = 30.87$	$F_{1,8} = 0.14$
			P =0.37	P < 0.001	P = 0.714
		Temperature	$F_{1,16} = 7.64$	$F_{1,16} = 246.06$	$F_{1,8} = 16.64$
			P = 0.013	P < 0.001	P = 0.0035
		Interaction	$F_{1,16} = 0.32$	$F_{1,16} = 2.48$	$F_{1,8} = 0.82$
			P = 0.58	P = 0.13	P = 0.39
Ai	ANOVA 2	Species	$F_{1,16} = 5.21$	$F_{1,16} = 0.22$	$F_{1,8} = 4.85$
			P = 0.036	P = 0.645	P = 0.0588
		Temperature	$F_{1,16} = 44.61$	$F_{1,16} = 19.44$	$F_{1,8} = 0.31$
			P < 0.001	P < 0.001	P = 0.593
		Interaction	$F_{1,16} = 0.019$	$F_{1,16} = 0.0037$	$F_{1,8} = 5.49$
			P = 0.892	P = 0.95	P = 0.0471

Table 4: Temperature effect on the Water Use Efficiency (WUE) (µmole CO_2/ mmole H_2O) of 2 buckwheat species. The plants were cultivated at either 21°C or 27°C for 8 weeks.

Species	Temperature	Weeks after treatment		
		Week 1	Week 4	Week 7
F. esculentum	21°C	0.4 ± 0.25	1.22 ± 0.12	1.38 ± 0.33
	27°C	1.16 ± 0.19	0.42 ± 0.12	1.23 ± 0.37
F. tataricum	21°C	0.54 ± 0.049	1.39 ± 0.26	1.45 ± 0.046
	27°C	1.44 ± 0.24	0.5 ± 0.25	0.58 ± 0.28
ANOVA 2	Species	$F_{1,16} = 6.03$	$F_{1,16} = 1.95$	$F_{1,8} = 2.94$
		$P = 0.026$	$P = 0.18$	$P = 0.12$
	Temperature	$F_{1,16} = 88.71$	$F_{1,16} = 88.41$	$F_{1,8} = 9.48$
		$P < 0.001$	$P < 0.001$	$P = 0.015$
	Interaction	$F_{1,16} = 0.67$	$F_{1,16} = 0.34$	$F_{1,8} = 4.6$
		$P = 0.42$	$P = 0.57$	$P = 0.064$



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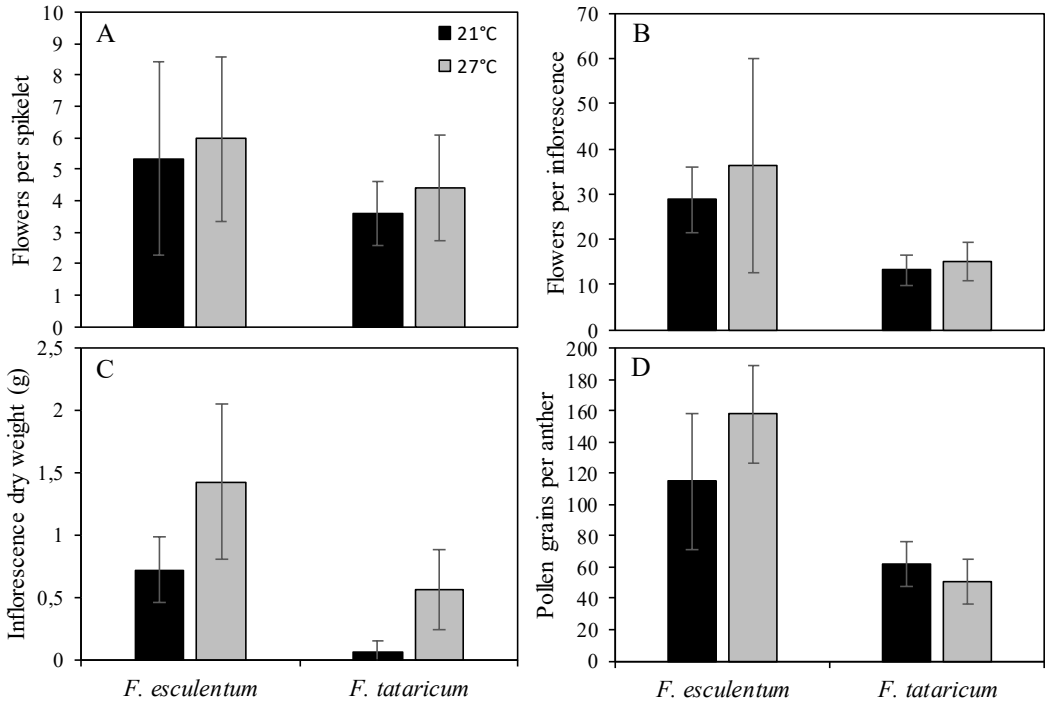

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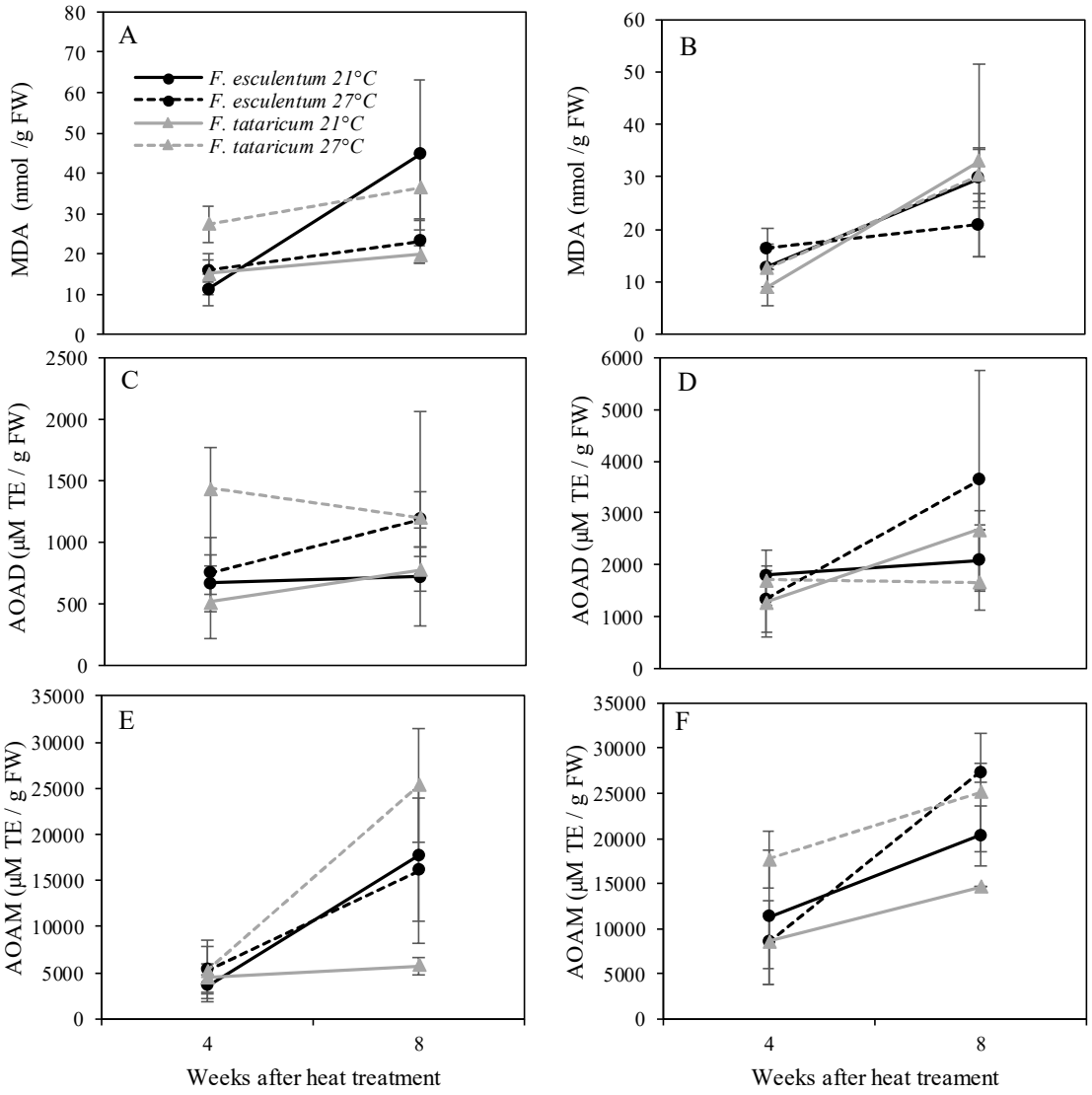

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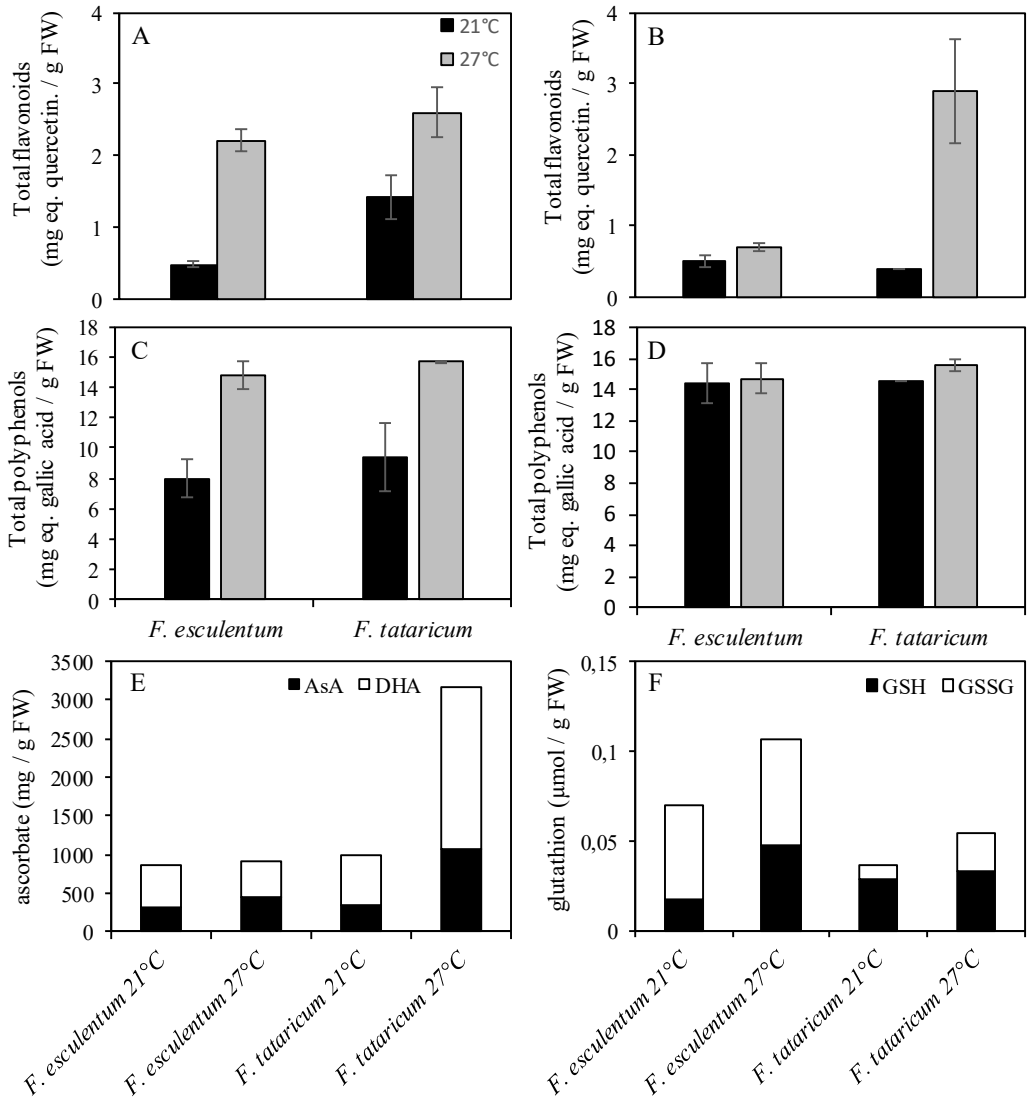

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